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# Characterization of the Human Visceral Adipose Tissue Secretome\*<sup>§</sup>

Gloria Alvarez-Llamas‡, Ewa Szalowska‡, Marcel P. de Vries‡, Desiree Weening‡, Karloes Landman‡, Annemieke Hoek§, Bruce H. R. Wolffenbuttel¶, Han Roelofsens‡, and Roel J. Vonk‡

Adipose tissue is an endocrine organ involved in storage and release of energy but also in regulation of energy metabolism in other organs via secretion of peptide and protein hormones (adipokines). Especially visceral adipose tissue has been implicated in the development of metabolic syndrome and type 2 diabetes. Factors secreted by the stromal-vascular fraction contribute to the secretome and modulate adipokine secretion by adipocytes. Therefore, we aimed at the characterization of the adipose tissue secretome rather than the adipocyte cell secretome. The presence of serum proteins and intracellular proteins from damaged cells, released during culture, may dramatically influence the dynamic range of the sample and thereby identification of secreted proteins. Part of the study was therefore dedicated to the influence of the culture setup on the quality of the final sample. Visceral adipose tissue was cultured in five experimental setups, and the quality of resulting samples was evaluated in terms of protein concentration and protein composition. The best setup involved one wash after the 1st h in culture followed by two or three additional washes within an 8-h period, starting after overnight culture. Thereafter tissue was maintained in culture for an additional 48–114 h to obtain the final sample. For the secretome experiment, explants were cultured in media containing L-[<sup>13</sup>C<sub>6</sub>, <sup>15</sup>N<sub>2</sub>]lysine to validate the origin of the identified proteins (adipose tissue- or serum-derived). In total, 259 proteins were identified with ≥99% confidence. 108 proteins contained a secretion signal peptide of which 70 incorporated the label and were considered secreted by adipose tissue. These proteins were classified into five categories according to function. This is the first study on the (human) adipose tissue secretome. The results of this study contribute to a better understanding of the role of adipose tissue in whole body energy metabolism and related diseases. *Molecular & Cellular Proteomics* 6: 589–600, 2007.

Adipose tissue is a key organ for the regulation of energy metabolism. Besides its function as an energy storage depot in the form of triglycerides, adipose tissue secretes a variety of peptide and protein hormones (adipokines) involved in the regulation of energy metabolism such as leptin, adiponectin, visfatin, retinol-binding protein-4, adipsin, tumor necrosis factor  $\alpha$  (TNF- $\alpha$ )<sup>1</sup> and interleukin 6 (1–3). Dysregulation of the production of adipokines and free fatty acids contributes to the pathogenesis of diseases associated with energy metabolism such as insulin resistance, metabolic syndrome, and type 2 diabetes. Especially visceral adipose tissue has been implicated in the development of these diseases (2–4). Therefore, more insight into the visceral adipose tissue secretome will contribute to a better understanding of its role in energy metabolism and related diseases and may lead to the discovery of unknown peptides/proteins involved in regulation of energy metabolism and new targets for therapy. Besides adipocytes, adipose tissue contains endothelial cells, macrophages, and fibroblasts (stromal fraction) that may modulate the overall peptide and protein secretion pattern of the tissue via cross-talk between the different cell types. For example, factors secreted by macrophages have been shown to induce changes in the secretion of adipokines, free fatty acids, and glucose uptake by 3T3-L1 adipocytes (5). These interactions between cells from the stromal fraction and adipocytes are necessary for physiological functions of adipose tissue, and deregulation of this cross-talk is regarded as an important mechanism leading to insulin resistance and type 2 diabetes (6–9). Therefore, the tissue secretome provides more relevant information for the *in vivo* situation than the adipocyte cell secretome. To date, no studies have been published on the adipose tissue secretome. Several studies investigated the human (10), mouse (11–14), and rat (15) adipose tissue proteome, mostly using a two-dimensional gel electrophoresis approach. Celis *et al.* (16) analyzed the human mammary adipose tissue proteome. The secretome from adipocyte cells has been investigated by Kratchmarova *et al.* (17) and Wang

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<sup>1</sup> The abbreviations used are: TNF- $\alpha$ , tumor necrosis factor  $\alpha$ ; PAI-1, plasminogen activator inhibitor-1; PTX3, pentraxin-related protein 3; FA, formic acid; SPARC, secreted protein acidic and rich in cysteine; MCSF, macrophage colony-stimulating factor; 2D, two-dimensional; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol; TMHMM, transmembrane hidden Markov model.

*et al.* (18). They studied changes in protein secretion during differentiation of the 3T3-L1 mouse preadipocyte cell line to adipocytes. In another study in isolated rat adipocytes, Chen *et al.* (19) identified 84 secreted proteins using 2D LC-MS/MS.

The major challenge for characterization of the adipose tissue secretome is the quality of the secretome sample. The presence of serum proteins inside the tissue pieces that slowly diffuse into the culture medium and the presence of intracellular proteins that are released from damaged cells due to the cutting of the tissue, necessary for culture, can dramatically influence the dynamic range of the sample and thereby the detection of the secreted proteins. Also the relevance of the identified proteins can be unclear if the source of the proteins (secreted, serum, or intracellular) is not clear due to a high level of contaminant proteins. This has been a problem in previous studies with tissue explants where *e.g.* adiponectin, secreted by the tissue, could not be reliably measured due to diffusion of serum adiponectin, as a second source of adiponectin, from the tissue into the culture medium (20). In addition, the duration of the culture influences the level of secreted proteins that accumulate in the medium but may also affect the function and breakdown of cells in the tissue. In view of secretome complexity and considering that adipokines are expected in low concentrations (ng/ml), the quality of the sample obtained from tissue culture is crucial to obtain high quality, relevant secretome data. Specific removal of high abundance serum proteins in biological samples has been described previously (21) but does not resolve the problem of contamination derived from intracellular proteins. Furthermore low abundance proteins that bind to the high abundance proteins may also be removed at the same time. Therefore, we did not consider this option but carefully evaluated the influence of the culture setup on the quality of the sample for secretome analysis. For this purpose, the influence of several culture setups that varied in the number of washes and the distribution of washes in time was investigated. Protein concentration, dynamic range, and composition of the resulting samples were then evaluated. From these experiments the best culture setup was chosen for further secretome characterization. Proteins were then identified by LC-MS/MS after SDS-PAGE fractionation. The resulting list of secreted proteins was validated by culturing adipose tissue in the presence of stable isotope-labeled L-lysine. Proteins that contain a signal peptide sequence and incorporate the label are derived from adipose tissue and not from an external source such as serum.

#### EXPERIMENTAL PROCEDURES

**Adipose Tissue Culture**—Human visceral adipose tissue explants were obtained from five women (age, 25–64 years; body mass index, 19–31) undergoing surgery for non-carcinogenic gynecologic disorders. For each culture setup (see Fig. 1), adipose tissue from one subject was used. The study had the approval of the local ethical committee.

The adipose tissue culture protocol is based on Fried and

Moustaid-Moussa (22). Briefly adipose tissue explants were transported from the operating room to the laboratory in transport buffer (PBS, 5.5 mM glucose, 50  $\mu$ g/ml gentamicin) at room temperature. The following procedures were carried out under a laminar flow hood using sterile equipment. Immediately upon arrival, the tissue was transferred to a Petri dish containing 20 ml of PBS and was finely minced in 20–80-mg pieces using scissors. The tissue pieces were extensively washed with 400 ml of PBS over a filter containing sterile cotton bandage fabric. Thereafter the tissue pieces were transferred to a 75-cm<sup>2</sup> culture flask containing 200 ml of PBS and were gently shaken for a short period. Next the contents of the flask were poured over the filter, and the tissue pieces were washed with 300 ml of warm PBS (37 °C). The tissue pieces were transferred to a tube containing 50 ml of PBS and centrifuged for 1 min at  $277 \times g$  at room temperature to remove red blood cells and debris. The tissue was then removed from the tube, and the weight was determined. 1 g of tissue was then placed in a Petri dish with 10 ml of M199 (Invitrogen) culture medium supplemented with 50  $\mu$ g/ml gentamicin. Adipose tissue pieces were cultured at 37 °C and 5% CO<sub>2</sub> following the different culture setups (Fig. 1). The final secretome sample and the media collected after every washing step in culture (Fig. 1) were stored at –80 °C.

**Sample Pretreatment**—9–10 ml of adipose tissue culture medium was concentrated 25–30-fold by ultrafiltration (Centriplus, 3-kDa cut-off, Millipore). The concentrated sample was used for total protein concentration measurement (Bradford assay, Bio-Rad), SELDI-TOF MS profiling, and protein identification by LC-MS/MS.

**SELDI-TOF MS Protein Profiling**—Spots of a CM10 (weak cation exchanger) ProteinChip® array (Ciphergen Biosystems, Fremont, CA), inserted into a bioprocessor, were preincubated twice with 200  $\mu$ l of binding buffer (100 mM ammonium acetate, 0.05% Triton, pH 4.0) for 5 min at room temperature with vigorous shaking. The buffer was then removed, and 4–12  $\mu$ l of concentrated sample (depending on protein concentration) were applied on every spot. Binding buffer was added to a total volume of 100  $\mu$ l/well ( $\mu$ g of protein was adjusted so that the same protein amount per spot was applied). After incubation for 30 min, the sample was removed, and the spots were washed three times with 200  $\mu$ l of binding buffer for 5 min followed by a wash with 200  $\mu$ l of ultrapure water. The water was removed, and the chip was allowed to air dry before applying  $2 \times 0.5 \mu$ l of 5 mg  $\alpha$ -cyano-4-hydroxycinnamic acid diethylamine salt dissolved in 1 ml of 50% ACN, 0.5% TFA. Mass analysis was performed in a ProteinChip reader (model PBS II, Ciphergen Biosystems) according to an automated data collection protocol. Calibration was performed with the All-in-One peptide mixture (Ciphergen Biosystems), and spectra were obtained in the mass range 3.5–100 kDa at several laser intensities (175–225).

**Label Experiment**—An adipose tissue explant from a patient undergoing surgery for uterus myomatosis (47 years old; body mass index, 22.9) was collected, cut, washed, and divided into two Petri dishes as described above. In this experiment, tissue was cultured from the start in lysine-free M199 medium (Invitrogen) to deplete lysine from other sources (serum). The medium was renewed after 1, 21.5, 25.5, and 29.5 h. In the last wash (time point, 29.5 h), one dish received normal M199 medium containing 70 mg/liter L-[<sup>12</sup>C<sub>6</sub>, <sup>14</sup>N<sub>2</sub>]lysine and 60 nM insulin. The other dish received M199 lysine-free medium containing 70 mg/liter labeled lysine (L-lysine: 2HCl; U-<sup>13</sup>C<sub>6</sub>, 98%; U-<sup>15</sup>N<sub>2</sub>, 98%; Cambridge Isotope Laboratories, Inc., Andover, MA) and 60 nM insulin. Tissue was maintained in culture for an additional 72 h. Thereafter media were collected and stored at –80 °C until analysis. Normal and labeled media were then mixed in 1:2 ratio and were concentrated by ultrafiltration before SDS-PAGE fractionation.

**Protein Identification by LC-MS/MS**—Proteins present in the con-

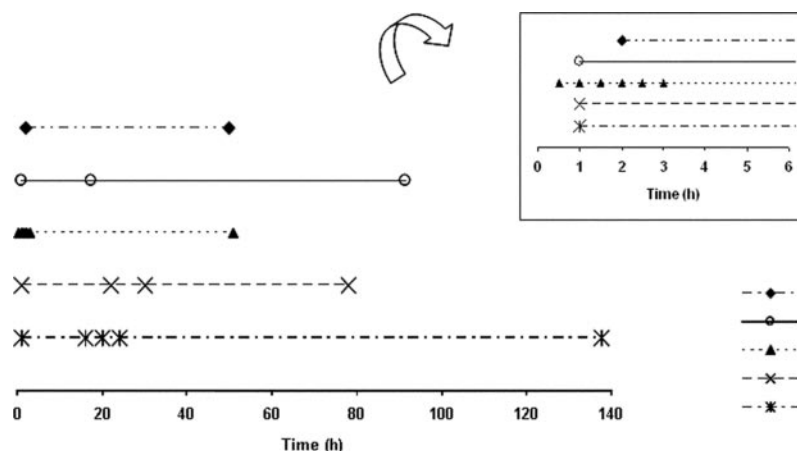


FIG. 1. Distribution of washing steps in time for the different culture setups (A–E) assayed. An inset is included for visualizing the distribution of washes at the beginning of the culture period (first 6 h).

centrated adipose tissue medium sample were fractionated by SDS-PAGE on a 4–12% bis-Tris gel with a MOPS buffer system according to the manufacturer's protocol (NuPAGE®-Novex, Invitrogen). Protein separation occurred for 50 min at 200 V, and visualization of bands was performed overnight by Coomassie Brilliant Blue G-250-based staining (PageBlue staining solution, Fermentas). The whole lane was excised into 28 bands, which were processed for tryptic digestion. Each band was cut into small pieces and stored at  $-20^{\circ}\text{C}$  until analysis. Then the pieces were washed in ultrapure water and dehydrated in ACN. In-gel reduction with dithiothreitol (for 1 h at  $60^{\circ}\text{C}$ ) and carbamidomethylation with iodoacetamide (for 45 min at room temperature in the dark) were performed. Gel pieces were subsequently washed with ultrapure water, 50% ACN, and pure ACN. Next  $0.1\text{ }\mu\text{g}$  of trypsin in 50 mM ammonium bicarbonate was added, and gel pieces were allowed to rehydrate on ice for 20 min. Digestion was carried out overnight at  $37^{\circ}\text{C}$ .

Separation of the resulting tryptic peptide mixtures was performed by nanoscale reversed-phase LC-MS/MS. The Agilent 1100 nano-flow/capillary LC system (Agilent, Palo Alto, CA) was equipped with a trapping column ( $5 \times 0.3\text{ mm C}_{18}$  reversed-phase) (Dionex/LC Packings, Amsterdam, The Netherlands) and a nanocolumn ( $150 \times 0.075\text{ mm, C}_{18}$  PepMap) (Dionex/LC Packings). Peptides mixtures were injected into the trapping column at a flow rate of  $10\text{ }\mu\text{L}/\text{min}$  (3% ACN, 0.1% FA). After 10 min the trapping column was switched into the nanoflow system, and the trapped peptides were separated using the nanocolumn at a flow rate of  $0.3\text{ }\mu\text{L}/\text{min}$  in a linear gradient elution from 95% A (3% ACN, 0.1% FA) to 50% B (97% ACN, 0.1% FA) in 50 min followed by an increase up to 80% B in 3 min. The eluting peptides were on-line electrosprayed into the QStar XL hybrid ESI quadrupole time-of-flight tandem mass spectrometer, ESI-qQ-TOF-MS/MS (Applied Biosystems, Framingham, MA; MDS Sciex, Concord, Ontario, Canada), provided with a nanospray source equipped with a New Objective ESI needle ( $10\text{-}\mu\text{m}$  tip diameter). Typical values for needle voltage were 2 kV in positive ion mode. Analyst QS 1.1 software (Applied Biosystems) was used for data acquisition in the positive ion mode typically with a selected mass range of 300–1200  $m/z$ . Peptides with +2 to +4 charge states were selected for tandem mass spectrometry, and the time of summation of MS/MS events was set to be 2 s. The three most abundant charged peptides above a 40 count threshold were selected for MS/MS and dynamically excluded for 40 s with 100-ppm mass tolerance.

ProID 1.1 software (Applied Biosystems) (23) was used to identify proteins from the mass spectrometric datasets according to the Swiss-Prot database (May 2005, ~181,000 entries). Mass tolerance

was set to 0.15 Da (MS) and 0.1 Da (MS/MS), and carbamidomethylation and methionine oxidation were chosen as modifications for database search.

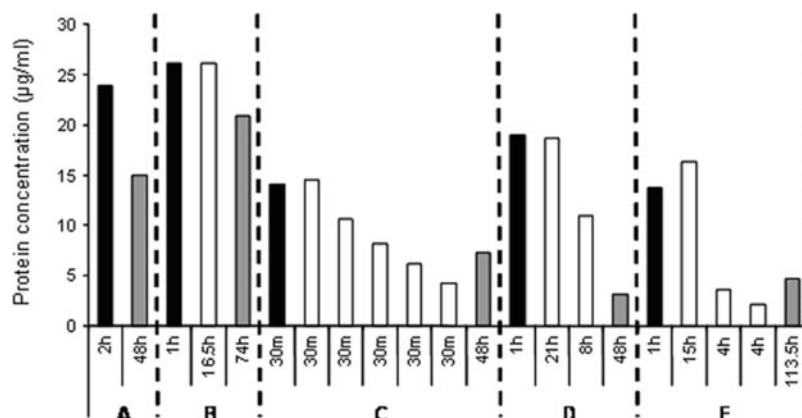
Classification of identified proteins in terms of secretion pathways was performed according to SecretomeP 2.0 Server (24). Those proteins with a signal peptide predicted by SignalP were considered as secreted proteins via a classical pathway (endoplasmic reticulum/Golgi-dependent pathway). If no signal peptide was predicted but the Neural Network score exceeded a value of 0.6, proteins were classified as secreted via the non-classical pathway. Transmembrane helices and location were predicted according to TMHMM Server (25). MS spectra of identified peptides that showed a lysine in the C terminus were searched for shifts of 8, 4, or 2.666  $m/z$  (singly, doubly, or triply charged ions, respectively). If a peptide incorporated the label, the derived protein was considered to be synthesized by adipose tissue.

## RESULTS

**Evaluation of Culture Setup and Sample Quality**—The standard adipose tissue culture protocol involves cutting of the tissue explants into small pieces followed by several washing steps to remove serum and intracellular proteins before culturing as described under “Experimental Procedures.” Because of the cutting, damaged cells will slowly lose their contents into the medium. Furthermore serum proteins still present in the tissue pieces will diffuse out during culture. Therefore, additional washing steps during culture were necessary to obtain a sample for secretome analyses containing mainly adipose tissue-derived secreted proteins. In preliminary studies we evaluated the protein composition of the adipose tissue medium, cultured for 48 h with one washing step after the 2nd h of culture (Fig. 1, setup A). After in-solution digestion of the final sample and LC-MS/MS analysis, 42 proteins were identified with  $\geq 95\%$  confidence (results not shown). According to the Human Protein Reference Database (HPRD) (26) none of them could be directly related to a protein secreted by adipose tissue. Typical serum proteins (albumin, hemoglobin, and transferrin) and intracellular proteins (actin, histones, and perilipin) dominated the secretome composition.



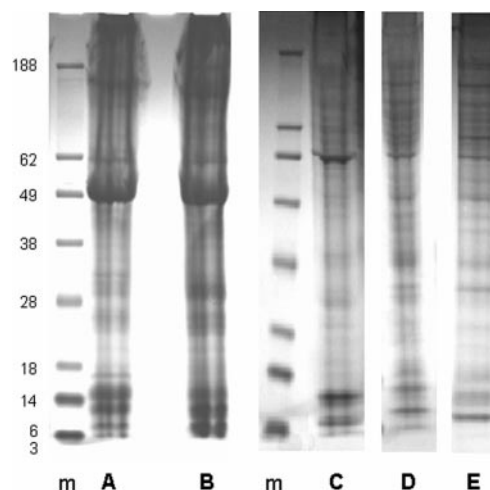
**FIG. 2. Total protein concentration measured in media collected from every washing step and in the final secretome sample.** On the x axis, consecutive time points are displayed per experiment, and every time point represents the culture time since the previous wash (periods of accumulation). The black bars represent the initial protein concentrations, and the gray bars refer to the final protein concentrations for every setup (A–E). Fig. 1 indicates the time points when the samples were collected.



To reduce the concentration of these high abundance contaminating proteins, five different culture setups were evaluated including the setup mentioned above as control (see Fig. 1). The number of washes (replacement with fresh media) during tissue culture, as well as their distribution in time, were varied to evaluate effects on the quality of the final sample for secretome analyses. Processing of the visceral adipose tissue explants, obtained from the five patients (A–E), was the same (described under “Experimental Procedures”). During culture, washing steps were performed by replacing media (10 ml) with 10 ml of fresh media according to the different schemes (A–E) depicted in Fig. 1. The media of the washing steps and the final secretome samples were collected for further analyses. Protein concentration was measured to evaluate the effectiveness of the different culture setups on removing (high abundance) serum and intracellular proteins. According to Fig. 2, protein concentrations in the final media samples were the lowest in setups D and E. This suggests that setups D and E were better in removing high abundance proteins when compared with setups A and B, whereas setup C gave an intermediate result.

The protein composition of the final samples obtained from each culture setup was investigated by SDS-PAGE (Fig. 3) and SELDI-TOF MS (Fig. 4). When comparing the lanes with samples of the indicated culture setups (Fig. 3), it was clear that samples from setups A and B showed less uniformity in intensity of bands than those of setups C, D, and E.

We also used SELDI profiling to monitor changes in the dynamic range of the samples that are expected to occur if high abundance contaminating proteins are removed more efficiently. High abundance proteins are likely to suppress ionization of low abundance proteins (27). As a consequence, with a certain laser energy, a lower total number of peaks can be expected in a sample with large concentration differences compared with a sample with small differences in concentration of individual proteins. We made use of this phenomenon to evaluate the dynamic range of the final samples resulting from the different culture setups (A–E). For this, spectra were obtained for every sample at four different laser intensities in the mass range from 3.5 to 17.5 kDa. Fig. 4.1 shows an



**FIG. 3. SDS-PAGE (12% bis-Tris gel) of final secretome samples obtained from the different culture setups as indicated in Fig. 1 (m, marker; A–E, final secretome samples).**

example of spectra obtained for the different setups with the lowest laser intensity (175). From these spectra it can already be deduced that samples of setups D and E showed considerably more peaks than samples from the other three setups at this laser intensity. Total peak number (signal/noise ratio >5) was also calculated from all spectra and plotted against laser intensity (Fig. 4.2). At laser intensity 190 still no maximum was reached in the number of detected peaks for setups A, B, and C, whereas the number of peaks that could be detected with setups D and E plateaued at laser intensities 180 and 185, respectively. This indicates that the dynamic range of samples D and E is lower than that of the other samples. We also monitored peak intensities of the  $\alpha$  and  $\beta$  chain of hemoglobin (predicted to be appearing at 15.1 and 15.9 kDa, respectively) and albumin (66 kDa) in the SELDI spectra that clearly showed a reduction in peak intensity (abundance) in spectra from samples D and E compared with A, B, and C (Fig. 4.3).

Combining results of these analyses, we conclude that one or two washing steps as used in setups A and B before isolation of the final medium sample are clearly not sufficient.

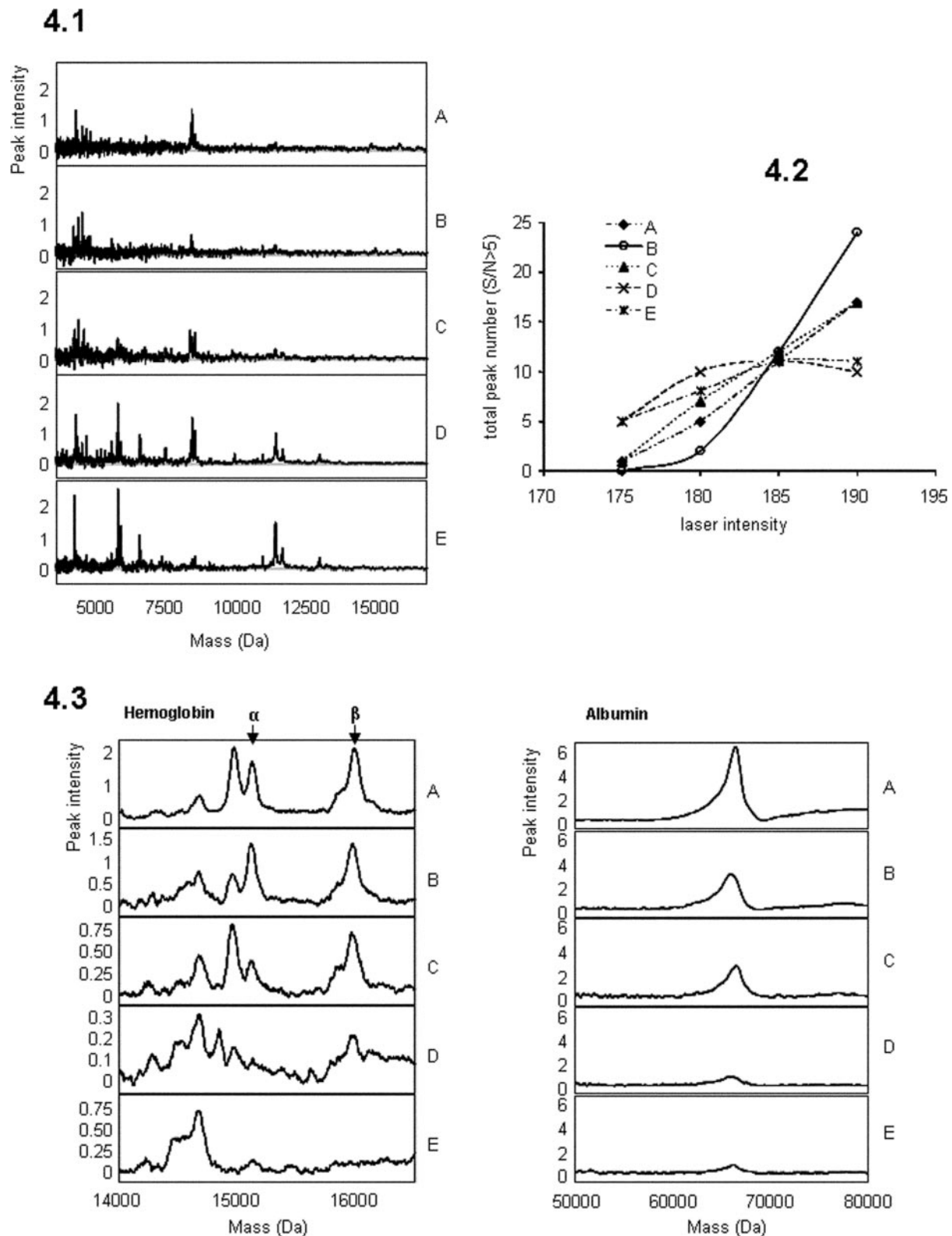


FIG. 4. 4.1, SELDI-TOF MS profiles (CM10 weak cation exchange chip) in the 3.5–17-kDa mass range at the lowest laser intensity (175). 4.2, graph of total peak number plotted against laser intensity by SELDI-TOF MS as an indication of the dynamic range of the final secretome samples. 4.3, SELDI-TOF MS spectra zoomed in at the mass range of hemoglobin and albumin (laser intensities, 185 and 225, respectively) for the five setups (A–E).

TABLE I

Secreted proteins (classical pathway; signal predicted by SignalP) that incorporated the label

The proteins were identified after SDS-PAGE fractionation and tryptic digest analysis by LC-MS/MS ( $\geq 99\%$  confidence). ECM, extracellular membrane; EGF, epidermal growth factor.

Accession no.	Protein name
<b>Signaling/regulatory function</b>	
gi 14916999	78-kDa glucose-regulated protein (GRP 78) (immunoglobulin heavy chain-binding protein)
gi 20137531	Adipocyte-derived leucine aminopeptidase (A-LAP) (ARTS-1) (aminopeptidase PILS)
gi 2493789	Adiponectin <sup>a,b,c</sup>
gi 117501	Calreticulin (CRP55) (calregulin) <sup>a</sup>
gi 1171064	Cell surface glycoprotein MUC18 (melanoma-associated antigen MUC18) <sup>d</sup>
gi 20177861	Complement C1q tumor necrosis factor-related protein 5
gi 23396772	Ectonucleotide pyrophosphatase/phosphodiesterase 2 (E-NPP 2)
gi 17865698	Endoplasmic (94-kDa glucose-regulated protein) (GRP94)
gi 23396609	Insulin-like growth factor-binding protein 7 (IGFBP-7) (IBP-7) (IGF-binding protein 7) <sup>a</sup>
gi 117558	Macrophage colony-stimulating factor-1 (CSF-1) (M-CSF) (M-CSF) <sup>d</sup>
gi 9297107	Neuropilin-1 (vascular endothelial cell growth factor 165 receptor) <sup>d</sup>
gi 118090	Peptidyl-prolyl cis-trans isomerase B (PPIase) (rotamase) (cyclophilin B)
gi 46576887	Periostin (PN) (osteoblast-specific factor 2) (OSF-2)
gi 3024715	Peroxiredoxin 4 (Prx-IV) (thioredoxin peroxidase AO372)
gi 20178323	Pigment epithelium-derived factor (PEDF) (EPC-1) <sup>a,c</sup>
gi 124096	Plasma protease C1 inhibitor (C1 Inh) <sup>a</sup>
gi 62298174	Plasma retinol-binding protein (PRBP) (RBP) <sup>a</sup>
gi 129576	PAI-1 (endothelial plasminogen activator inhibitor) <sup>a</sup>
gi 401413	von Willebrand factor precursor (vWF)
<b>ECM</b>	
gi 24212664	Basement membrane-specific heparan sulfate proteoglycan core protein (HSPG) (perlecan)
gi 115269	Collagen $\alpha$ 1(I) chain <sup>c</sup>
gi 115306	Collagen $\alpha$ 1(III) chain <sup>b,c</sup>
gi 13878903	Collagen $\alpha$ 1(VI) chain <sup>b,c</sup>
gi 62901508	Collagen $\alpha$ 1(XIV) chain (undulin)
gi 728996	Collagen $\alpha$ 1(XV) chain
gi 45644997	Collagen $\alpha$ 1(XVIII) chain
gi 8039779	Collagen $\alpha$ 2(I) chain <sup>c</sup>
gi 115349	Collagen $\alpha$ 2(IV) chain <sup>a,c</sup>
gi 27808647	Collagen $\alpha$ 2(VI) chain <sup>b,c</sup>
gi 5921193	Collagen $\alpha$ 3(VI) chain <sup>b</sup>
gi 62510689	EGF-containing fibulin-like extracellular matrix protein 1 (fibulin-3)
gi 2506872	Fibronectin (FN) (cold-insoluble globulin)
gi 47115668	Galectin-3-binding protein (lectin galactoside-binding soluble 3-binding protein)
gi 121116	Gelsolin (actin-depolymerizing factor) (ADF) (brevin) <sup>a,b,c</sup>
gi 20141592	Laminin $\alpha$ -4 chain
gi 126366	Laminin $\beta$ -1 chain (laminin B1 chain) <sup>a</sup>
gi 126369	Laminin $\gamma$ -1 chain (laminin B2 chain) <sup>a</sup>
gi 62298084	Matrilin-2
gi 2506403	Microfibril-associated glycoprotein 4
gi 128199	Nidogen (entactin) <sup>a,b</sup>
gi 52783472	Spondin-1 (F-spondin) (vascular smooth muscle cell growth-promoting factor)
gi 3915888	Tenascin (TN) (hexabrachion) (cytotactin) (neuronectin) (GMEM)
gi 135717	Thrombospondin-1 <sup>a</sup>
gi 549136	Thrombospondin-2
gi 2498193	Transforming growth factor- $\beta$ -induced protein IG-H3 ( $\beta$ IG-H3)
gi 2506816	Versican core protein (large fibroblast proteoglycan)
<b>Immune function</b>	
gi 115205	Complement C1s subcomponent (C1 esterase) <sup>a</sup>
gi 38257345	Complement C2 (C3/C5 convertase) <sup>a</sup>
gi 116594	Complement C3 <sup>b,c</sup>
gi 20141171	Complement C4 <sup>a</sup>
gi 61252057	Complement component C7
gi 584908	Complement factor B (C3/C5 convertase) (properdin factor B) <sup>a</sup>
gi 3915626	Complement factor D (C3 convertase activator) (properdin factor D) (adipsin) <sup>a,b,c</sup>
gi 48428995	Lysozyme C (1,4- $\beta$ -N-acetylmuramidase C)

TABLE I—continued

Accession no.	Protein name
Involved in degradation	
gi 116856	72-kDa type IV collagenase (matrix metalloproteinase-2) (MMP-2) <sup>a,b,c</sup>
gi 112911	$\alpha_2$ -Macroglobulin ( $\alpha_2$ -M)
gi 115711	Cathepsin B (cathepsin B1) (APP secretase) <sup>a</sup>
gi 115717	Cathepsin D <sup>a</sup>
gi 115741	Cathepsin L (major excreted protein) (MEP)
gi 544413	Chitinase-3-like protein 1 (cartilage glycoprotein-39)
gi 116852	Interstitial collagenase (matrix metalloproteinase-1) (MMP-1) (fibroblast collagenase)
gi 116863	Matrix metalloproteinase-9 (MMP-9) (92-kDa type IV collagenase) (92-kDa gelatinase) <sup>a</sup>
gi 135850	Metalloproteinase inhibitor 1 (TIMP-1) (erythroid potentiating activity) (EPA) <sup>a</sup>
gi 6919941	Procollagen C-proteinase enhancer protein (PCPE) <sup>c</sup>
Other functions	
gi 119576	Liver carboxylesterase 1 (acyl-coenzyme A:cholesterol acyltransferase)
gi 585223	Plasma glutathione peroxidase (GSHPx-P)
gi 61221730	Protein C19orf10 (stromal cell-derived growth factor SF20) (interleukin-25) <sup>c</sup>
gi 2507461	Protein-disulfide isomerase A3 (disulfide isomerase ER-60) (ERp60)
gi 2507460	Protein-disulfide isomerase (PDI) (prolyl 4-hydroxylase $\beta$ subunit)
gi 136191	Serotransferrin (transferrin) (siderophilin) ( $\beta$ -1-metal-binding globulin) <sup>a</sup>

<sup>a</sup> Secreted proteins also identified in Ref. 19.

<sup>b</sup> Secreted proteins also identified in Ref. 17.

<sup>c</sup> Secreted proteins also identified in Ref. 18.

<sup>d</sup> Signal peptide predicted by SignalP (24) but also transmembrane helices predicted by transmembrane hidden Markov model (TMHMM) Server (25) (more than one helix or one helix located more than 40 amino acids away from the N terminus of the protein).

Setups D and E contained three or four washing steps, respectively, distributed over a period of 24 h before the final sample was obtained. This clearly gave better results also when compared with setup C. Here six washing steps were applied but all in the beginning of the tissue culture period. Based on these analyses we conclude that culture protocols D and E performed best.

**Secretome Characterization**—After the optimal culture setup was established, this protocol was used for characterization of the secretome. To determine whether identified proteins are secreted by adipose tissue or are derived from serum that may still be present as a contaminant of the sample, a labeling experiment was carried out. In this qualitative approach, an adipose tissue explant was divided into two dishes. After the washing steps were performed in lysine-free media (see “Experimental Procedures”), the tissue was cultured for an additional 72 h. One dish contained medium supplemented with L-[<sup>13</sup>C<sub>6</sub>, <sup>15</sup>N<sub>2</sub>]lysine, and the other dish contained normal medium containing L-[<sup>12</sup>C<sub>6</sub>, <sup>14</sup>N<sub>2</sub>]lysine. To facilitate detection of label incorporation by mass spectrometry, media from both dishes were mixed in a 1:2 ratio in favor of the stable isotope label. After concentration, the sample was fractionated by SDS-PAGE. The lane was excised in 28 bands for tryptic digestion, and digests were analyzed by LC-MS/MS for protein identification as described under “Experimental Procedures.” The complete list of identified proteins and peptide sequences obtained from the MS/MS data can be found in the Supplemental Table C. Peptide confidences >5 are shown for each identified protein. Confidence scores generated by the ProID software are explained in a study by Tang *et al.* (23). By this approach, a total of 297

proteins could be identified with  $\geq 95\%$  confidence. Within this group, 259 proteins were identified with  $\geq 99\%$  confidence. Proteins identified with the highest confidence score ( $\geq 99\%$ ) were analyzed for the presence of a signal peptide using the SecretomeP 2.0 Server to determine whether they were secreted. This analyses revealed that 108 of 259 proteins were secreted following a classical pathway (endoplasmic reticulum/Golgi-dependent pathway) (see Tables I and II). For all these proteins a signal peptide was predicted by SignalP. Some of them (marked with Footnote d in Tables I and II) contained transmembrane helices located more than 40 amino acids away from the N terminus according to the TMHMM Server. This implies that they were anchored to the membrane. However, because a signal peptide was predicted we considered them as secreted. The possibility exists that they were released from the cell via cleavage of the extracellular part of the protein. Proteins identified with  $\geq 99\%$  confidence that contained a signal peptide were classified according to whether or not they incorporated the label (Tables I and II). Of 108 proteins containing a signal peptide, label incorporation could be confirmed for 70 of them (see Table I). These proteins were considered genuine adipose tissue-secreted proteins. For this, the MS spectra of the corresponding peptides were manually checked for mass shifts due to label incorporation as described under “Experimental Procedures.” Some proteins were only identified by peptides ending on an arginine at the C terminus (no lysine present). This leaves open the possibility that they are derived from adipose tissue although the label incorporation could not be detected (see first group of non-labeled proteins in Table II).

As an example, Fig. 5 shows two MS spectra obtained for



TABLE II

*Secreted proteins (classical pathway; signal predicted by SignalP) that did not incorporate the label*

The proteins were identified after SDS-PAGE fractionation and tryptic digest analysis by LC-MS/MS ( $\geq 99\%$  confidence). EGF, epidermal growth factor.

Accession no.	Protein name
Identification based on only Arg-ending peptides	
gi 12643637	ADAMTS-4 (a disintegrin and metalloproteinase with thrombospondin motifs 4)
gi 416746	Azurocidin (cationic antimicrobial protein CAP37) (heparin-binding protein)
gi 23396490	Calsyntenin-1 <sup>a</sup>
gi 12643324	Cathepsin Z
gi 116533	Clusterin (complement-associated protein SP-40,40) (complement cytolysis inhibitor) <sup>b</sup>
gi 3023630	Cystatin C <sup>b,c</sup>
gi 462007	Dermatopontin (tyrosine-rich acidic matrix protein) (TRAMP)
gi 37537873	EMILIN 1 (elastin microfibril interface-located protein 1)
gi 134635	Extracellular superoxide dismutase (Cu-Zn) (EC-SOD) <sup>d</sup>
gi 30581038	Fibulin-1
gi 41017299	Latent transforming growth factor- $\beta$ -binding protein 2 (LTBP-2)
gi 126279	Leukemia inhibitory factor (LIF) (differentiation-stimulating factor) (D factor)
gi 119292	Leukocyte elastase (neutrophil elastase) (PMN elastase) (bone marrow serine protease)
gi 20141203	Monocyte differentiation antigen CD14 (myeloid cell-specific leucine-rich glycoprotein) <sup>b</sup>
gi 129825	Myeloperoxidase (MPO)
gi 8928569	Nidogen-2 (NID-2) (osteonidogen)
gi 50400889	Olfactomedin-like protein 1
gi 1346908	Pentraxin-related protein PTX3 (pentaxin-related protein PTX3)
gi 62900717	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 3 (lysyl hydroxylase 3) (LH3)
gi 41017497	Prostaglandin-H <sub>2</sub> D-isomerase (lipocalin-type prostaglandin-D synthase)
gi 2501205	Protein-disulfide isomerase A6 (protein-disulfide isomerase P5)
gi 129283	SPARC (osteonectin) <sup>b,c,d</sup>
gi 52783469	Spondin-2 (mindin)
gi 3334154	Stanniocalcin 1 (STC-1)
gi 1351316	Tumor necrosis factor-inducible protein TSG-6 (TNF-stimulated gene 6 protein)
gi 13432109	Vascular endothelial-cadherin (VE-cadherin) (cadherin-5) (7B4 antigen) (CD144 antigen) <sup>a</sup>
Identification based on Lys-ending peptides	
gi 57015285	Citrate synthase, mitochondrial precursor
gi 3182940	Collagen $\alpha$ 1(XII) chain
gi 12643876	Fibulin-5 (FIBL-5) (developmental arteries and neural crest EGF-like protein)
gi 6175096	Lactotransferrin (lactoferrin)
gi 1708851	Laminin $\beta$ -2 chain (S-laminin) (laminin B1s chain)
gi 20141464	Lumican (keratan sulfate proteoglycan lumican) (KSPG lumican)
gi 2501336	Membrane copper amine oxidase (vascular adhesion protein-1) (VAP-1)
gi 129078	Mimecan (osteoglycin) (osteoinductive factor)
gi 1171700	Neutrophil gelatinase-associated lipocalin (NGAL) (P25) <sup>c</sup>
gi 51701718	Plexin B2 (MM1)
gi 113576	Serum albumin <sup>b</sup>
gi 9087217	Tenascin-X (TN-X)

<sup>a</sup> Signal peptide predicted by SignalP (24) but also transmembrane helices predicted by TMHMM Server (25) (more than one helix or one helix located more than 40 amino acids away from the N terminus of the protein).

<sup>b</sup> Secreted proteins also identified in Ref. 19.

<sup>c</sup> Secreted proteins also identified in Ref. 17.

<sup>d</sup> Secreted proteins also identified in Ref. 18.

two different proteins, endoplasmin (Fig. 5A) and plasminogen activator inhibitor-1 (PAI-1) (Fig. 5B). The mass shift was clearly seen in both cases (+4  $m/z$ ), although the different relative peak intensities for the non-labeled and labeled peptides point to different incorporation rates of the label for these two proteins. Fig. 5C shows the MS/MS spectra from both the original and the labeled peptides from PAI-1 where also a mass shift of +8  $m/z$  was obvious.

Secreted proteins were classified into groups with similar functions (Table I). For this, information was obtained from

Swiss-Prot and SOURCE (source.stanford.edu) databases. The criteria for classification are not always uniform because many proteins have more than one function that may place them in more than one functional category. In this case, what was considered as the main function of the protein was used for classification in one of the five categories: signaling/regulatory, extracellular matrix, immune function, degradation, and other.

In Supplemental Table A, non-secreted proteins (no signal peptide predicted by SignalP according to SecretomeP) and

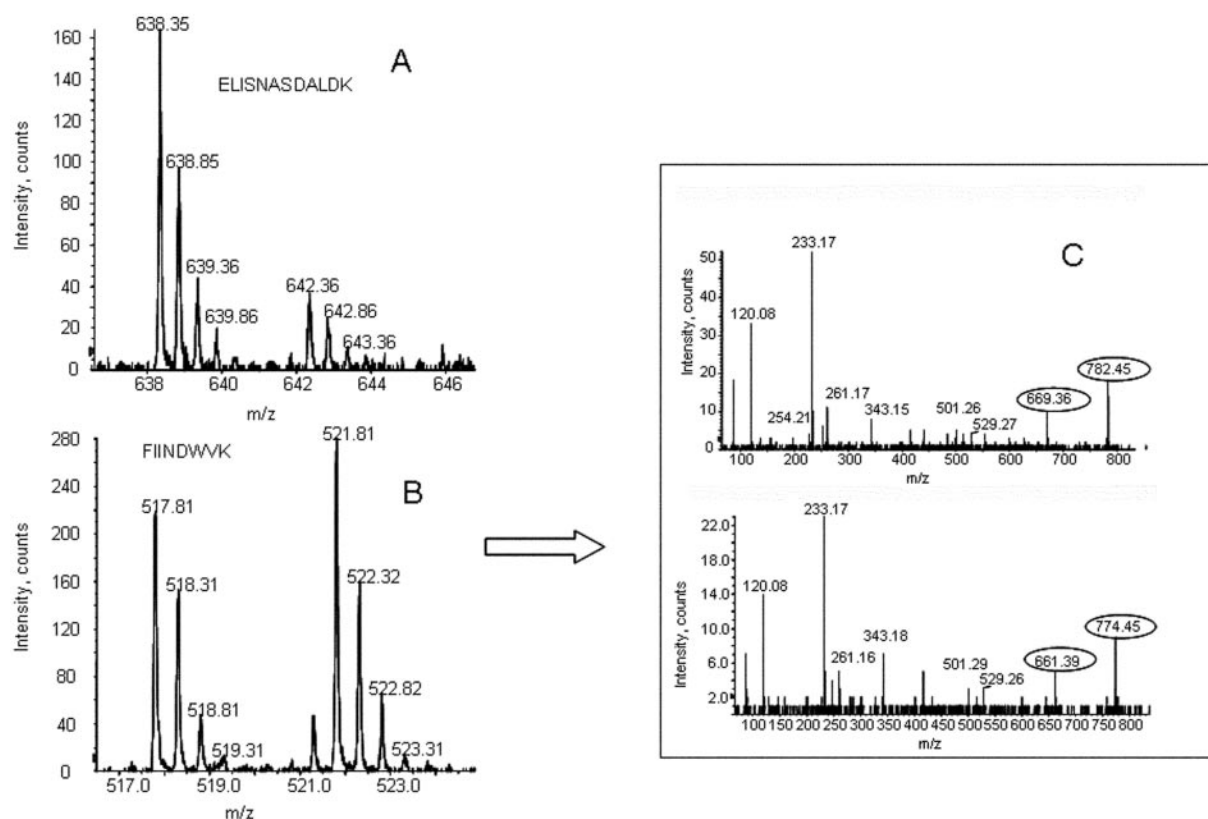


FIG. 5. **Examples of different incorporation ratios in labeled proteins.** A, TOF MS spectra of endoplasmic peptide; B, TOF MS spectra of PAI-1 peptide; C, MS/MS spectra of both original and labeled peptides in PAI-1.

non-classically secreted proteins (no signal peptide predicted by SignalP but Neural Network score  $>0.6$ , marked as °) are shown. In Supplemental Table B, classically secreted proteins identified with confidence between 95 and 99% are shown.

#### DISCUSSION

Adipose tissue is recognized as an important organ for the regulation of the whole-body energy metabolism through the secretion of adipokines. Although major adipokines such as leptin and adiponectin have been shown to be produced by adipocytes, also other cell types that are part of the adipose tissue produce adipokines or influence production of adipokines by adipocytes. Fain *et al.* (28) showed that the majority of adipokines measured in their study were released by non-adipocyte cells in the tissue. This fact points out the relevance of evaluating the adipose tissue secretome rather than the adipocyte cell secretome. Here we describe the first proteomics study on the adipose tissue secretome. A major issue in characterizing the adipose tissue secretome is that protein composition of the adipose tissue culture media is highly dependent on the way the tissue culture is performed. This not only has implications for proteomics studies but also for other studies where adipose tissue culture is used to study individual adipokines by ELISA because these peptide and protein hormones are also present in serum and in the intra-

cellular protein fraction, which, as we demonstrate, are the main sources of secretome contamination in adipose tissue culture. Therefore, we established a tissue culture protocol that minimizes contamination of the secretome with serum-derived and intracellular proteins. After analyses of five different culture setups we conclude that a tissue culture protocol with one wash after the 1st h in culture and two or three additional washes after overnight culture within a period of 8 h followed by a 48–114-h incubation period (setups D and E, Fig. 1) provides the optimal culture protocol to obtain a high quality sample for secretome analyses. With this setup, total protein concentration was reduced from  $\sim 17 \mu\text{g/ml}$  to around  $4 \mu\text{g/ml}$  (setups D and E) at the end of the culture period (Fig. 2). This reduction is considerably higher than that observed for setups A and B (from about  $25 \mu\text{g/ml}$  to about  $18 \mu\text{g/ml}$ ). Albumin and hemoglobin (serum contaminating proteins) seemed to be highly reduced in setups D and E (Fig. 4.3), and the dynamic range of the sample had decreased considerably (Figs. 3 and 4.2) compared with setups A and B.

This new, improved culture protocol was used to characterize the human adipose tissue secretome. Proteins were identified, and the presence of a signal peptide was investigated to distinguish those that are secreted in the medium from those that are intracellular proteins, derived from damaged cells. Furthermore a metabolic labeling approach was

designed to differentiate between secreted proteins derived from adipose tissue and serum proteins that still may be present in low levels as a contaminant of the sample. Proteins that contained a signal peptide and incorporated the label were considered as genuinely secreted by adipose tissue and not coming from an external source (serum or intracellular origin). With this strategy, different incorporation rates of the label into the proteins were noticed that can be related to different turnover rates. In summary, a total of 297 proteins were identified with  $\geq 95\%$  confidence. 259 proteins were identified with  $\geq 99\%$ , among which were 108 secreted proteins (Tables I and II). Of 108 secreted proteins, 70 proteins incorporated the label (Table I), *i.e.* adiponectin, adipsin, gelsolin, macrophage colony-stimulating factor-1 (MCSF), pigment epithelium-derived factor, plasma retinol-binding protein, and PAI-1 among others. Interleukin-6 was also detected, showing a clear incorporation of the label, although it was identified with  $\geq 95\%$  confidence, (Supplemental Table B). Adipocyte fatty acid-binding protein was identified as a non-classically secreted protein (Supplemental Table A) with label incorporation in agreement with a recent study by Xu *et al.* (29) where they claimed that adipocyte fatty acid-binding protein is a circulating biomarker released from adipocytes into the bloodstream, closely associated with obesity and metabolic syndrome. For 38 proteins containing a signal peptide the label could not be detected (Table II). However, 26 of these were identified with peptides ending on an arginine in the C terminus (no lysine present), implying that it is still possible that these proteins are secreted by adipose tissue although label incorporation could not be confirmed (*i.e.* leukemia inhibitory factor, pentraxin-related protein 3 (PTX3), and secreted protein acidic and rich in cysteine (SPARC)). Finally the label could not be detected in a group of 12 proteins even though their peptides showed a lysine in the C terminus. This fact may be due to a low incorporation rate or because the protein was derived from serum, *e.g.* albumin is part of this latter group (Table II).

In Supplemental Table A, non-secreted proteins (intracellular) or proteins predicted to be secreted via a non-classical pathway are shown (151 proteins in total). Non-secreted proteins, such as actin, histones, catalase, and proteasome subunits, are intracellular proteins that may be derived from damaged cells in culture or from cleaved fragments of membrane proteins. This together with the fact that albumin was still detected indicates that, although with the improved tissue culture setup sample quality had improved considerably, it was not completely free of (high abundance) serum and intracellular proteins. Nevertheless the dynamic range of the sample improved considerably allowing a much more sensitive secretome identification. The level of sensitivity that was reached with the described procedure was in the low ng/ml range because adiponectin, which was identified with  $\geq 99\%$  confidence, reached concentrations of around 10–20 ng/ml at the end of the culture period as determined by ELISA in similar experiments.

Three studies have been published thus far on the adipocyte cell secretome. We compared the results of those studies with our results on the human visceral adipose tissue secretome. Chen *et al.* (19) identified 84 proteins secreted by isolated rat adipocytes cells using 2D LC-MS/MS. Of these proteins 29 were also identified in the present study (as indicated in Tables I and II). Wang *et al.* (18) identified 27 proteins that were secreted during mouse 3T3-L1 adipocyte cell differentiation by two-dimensional gel electrophoresis-MS/MS. 16 proteins were also identified in our study (as indicated in Tables I and II). Of 20 secreted proteins identified by Kratchmarova *et al.* (17) during differentiation of 3T3-L1 preadipocytes by SDS-PAGE and LC-MS/MS, 13 were also found in this study (as indicated in Tables I and II). Only five proteins are shared between the three adipocyte cell studies and the present study on adipose tissue. These proteins are adiponectin, SPARC, gelsolin, adipsin, and matrix metalloproteinase-2. 68 proteins (classically secreted) identified in the present study were not found in the other three studies. This may be explained by the different starting materials (cells or tissue), the different proteomics approaches that were followed, and the different origins of the material (rat, mouse, or human).

As mentioned before, although the adipocyte is the major cell type in adipose tissue, this study shows that a substantial number of proteins secreted by the tissue are released by other cell types such as macrophages and endothelial cells. In particular, some of the secreted proteins identified in the present study were also identified in human macrophages by a 2D gel approach (30). That is the case for endoplasmic reticulum chaperones, protein-disulfide isomerase, protein-disulfide isomerase A3, calreticulin, cathepsin D, and peroxiredoxin 4 among others. In the same way, PTX3 mRNA is expressed in the stromal-vascular fraction of adipose tissue but not in fully differentiated adipocytes; it plays a role in the regulation of resistance to pathogens and inflammatory reactions, and the PTX3 gene can be induced in adipocytes by TNF- $\alpha$  (31). Taking into account what has been reported previously (1, 7, 32–35) on proteins secreted by adipose tissue, we estimate that in this study we identified 48 new proteins as being secreted by adipose tissue.

The functional classification of the identified secreted proteins, as shown in Table I, indicates that 39% of the proteins are involved in the modulation of the extracellular matrix. An example from this group is perlecan. It is a secreted proteoglycan, widely distributed as part of the basement membrane, that may bind lipoprotein lipase and is localized close to the cell surface so that it can participate in triacylglycerol hydrolysis (36). Another example from this group is versican core protein that plays a role in intercellular signaling and in connecting cells with the extracellular matrix. It may also take part in the regulation of cell motility, growth, and differentiation (37). The second largest group (27%) consists of proteins involved in signaling and regulation. MCSF is part of this

group. It promotes human adipose tissue hyperplasia, it is up-regulated under conditions favoring adipose tissue growth (obesity), and it is down-regulated by TNF- $\alpha$  (38). Another example is neuropilin-1, which is expressed by adipocytes and is involved in regulation of angiogenesis (39). Proteins classified as being involved in degradation (14%) are e.g. cathepsins B and D. These are lysosomal proteases, but they can also be secreted. The fact that a large portion of the identified proteins are involved in the modulation of extracellular matrix, protein degradation, and regulation of cellular processes indicates that adipose tissue is a very actively dividing tissue that is probably related to the demand to store energy in the form of triglycerides. Therefore, the tissue has to be flexible to increase or decrease storage capacity.

In conclusion, adipose tissue culture setup has a strong influence on the quality of the sample and on detection of secreted proteins. We show here that proteins secreted from adipose tissue can be unequivocally identified by a qualitative labeling approach that has the capability to distinguish the source of relevant proteins. For the future it will be interesting to compare adipose tissue secretomes from lean and obese people to determine differences in protein expression that may lead to the discovery of mechanisms involved in insulin resistance and type 2 diabetes. For this, a quantitative labeling approach should be developed. We are currently working on this topic.

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